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Supplemental Assay Method for Potency Testing of Inactivated Rabies Vaccine in Mice Using the National Institutes of Health Test

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Table of Contents

Supplemental Assay Method for Potency Testing of Inactivated Rabies Vaccine in Mice Using the National Institutes of Health Test

1. Introduction

This Supplemental Assay Method (SAM) describes testing inactivated rabies vaccines for relative potency (RP). The method uses immunized mice to measure protection following a challenge with rabies. The RP is determined by comparing the Test Vaccine against a standardized reference vaccine. This standard National Institutes of Health (NIH) test method is described in *Laboratory Techniques in Rabies*, fourth edition, edited by F. X. Meslin, M. M. Kaplan, and H. Koprowski, 1996, published by the World Health Organization (WHO), Geneva, Switzerland.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.2 Centrifuge with rotor (Model TJ-6, Beckman Instruments Inc.)

2.1.3 Water bath

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 CF-1 female mice weighing 13-15 g

2.2.2 Rabies mouse challenge: Challenge virus standard (CVS) [available from the Center for Veterinary Biologics (CVB)]

2.2.3 Veterinary rabies reference vaccine (VRRV) [available from CVB]

2.2.4 Centrifuge tube, 15-ml

2.2.5 Glass tube, sterile, 16 x 125-mm with Morton closure

2.2.6 Flanged stopper, sterile

2.2.7 Syringe, 3-ml and needle, 20-gauge x 1 1/2-inch

2.2.8 Tuberculin syringe, 0.5-ml and needle, 26-gauge x 3/8-inch

2.2.9 Syringe, 10-ml and needle, 25-gauge x 5/8-inch

2.2.10 Phosphate buffered saline (PBS)

2.2.10.1 0.804 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2.2.10.2 0.136 g potassium phosphate, monobasic, monohydrate (KH_2PO_4)

2.2.10.3 8.5 g sodium chloride (NaCl)

2.2.10.4 Q.S. to 1000 ml with deionized water (DI).

2.2.10.5 Adjust pH to 7.6 with 0.1 N sodium hydroxide (NaOH).

2.2.10.6 Sterilize through a 0.22-µm filter.

2.2.10.7 Store at 2°- 7°C.

2.2.11 7.5% Sodium Bicarbonate

2.2.11.1 7.5 g sodium bicarbonate ($NaHCO_3$)

2.2.11.2 Q.S. to 100 ml with DI

2.2.11.3 Store at room temperature.

2.2.12 CVS Diluent

2.2.12.1 20 ml heat-inactivated, rabies antibody-free horse serum

2.2.12.2 500,000 units penicillin

2.2.12.3 1 g streptomycin

2.2.12.4 Q.S. to 1000 ml with DI.

2.2.12.5 Adjust pH to 7.6 with 7.5% Sodium Bicarbonate.

2.2.12.6 Sterilize through a 0.22-µm filter.

2.2.12.7 Store at 2°- 7°C; use within 12 weeks.

2.2.13 Pipettes, 2-ml, 5-ml, 10-ml and 25-ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in laboratory dilution techniques, the handling and discarding of potential human pathogens, and the handling and inoculation of mice.

CAUTION: Live rabies is a potential deadly human pathogen! To work with live rabies, personnel must be vaccinated for rabies and monitored for a minimum rabies titer acceptable to appropriate human health officials. The use of appropriate biological safety cabinets is required for dilution of challenge material. All discarded challenge materials should be considered potentially infective and handled in a manner consistent with safe laboratory practices and in accordance with the recommendations of the Centers for Disease Control and Prevention and the NIH. All challenged mice should be disposed of in an appropriate manner.

3.2 Preparation of equipment/instrumentation

On the day of the second vaccination and on the day of the challenge, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 On the day of initial vaccination, using a syringe, rehydrate the VRRV with sterile DI according to the supplied Center for Veterinary Biologics (CVB) Reagent Data Sheet.

3.3.1.1 Make an initial calibration dilution of the VRRV in PBS according to the supplied CVB Reagent Data Sheet.

3.3.1.2 The starting dilution of the VRRV is at your discretion as long as the validity requirements in **Section 5.1** are met. An example of a fivefold dilution series starting at 1:10 is as follows:

1. Using a 25-ml pipette, dispense 13.5 ml of PBS into a 16 x 150-mm glass tube with a Morton closure; label the tube 1:10. Replace the Morton closure with a flanged stopper.

2. Using a 3-ml syringe with a 20-gauge needle, transfer 1.5 ml of the VRRV (**Section 3.3.1.1**) into the 1:10 labeled tube; mix by vortexing.

3. Using a 25-ml pipette, dispense 12.0 ml of PBS into 3, 16 x 150-mm glass tubes with flanged stoppers; label tubes 1:50, 1:250, and 1:1250.

4. With a new 3-ml syringe and a 20-gauge needle, transfer 3.0 ml from the 1:10 tube to the 1:50 tube; mix by vortexing.

5. Repeat **Section 3.3.1.2(4)** for the remaining tubes, transferring 3.0 ml from the previous tube to the next tube.

6. The VRRV dilution tubes are maintained in a bucket of ice until inoculated into mice. The diluted VRRV must be used within 3 hours of preparation.

7. After completing the dilutions, freeze the remaining reconstituted VRRV from **Section 3.3.1**, in the original container at -70°± 5°C for the second vaccination.

3.3.1.3 Preparation of VRRV for the second vaccination (7 ± 1 days postinoculation)

1. Rapidly thaw the frozen reconstituted VRRV in a 36°± 2°C water bath.

2. Repeat **Sections 3.3.1.2(1) through 3.3.1.2(6)**.

3.3.2 Preparation of Working CVS on the day of challenge

3.3.2.1 Rapidly thaw CVS in a 36°± 2°C water bath.

3.3.2.2 Transfer CVS to a 15-ml centrifuge tube with a 2-ml pipette.

3.3.2.3 Centrifuge CVS at 200 X *g* at room temperature for 10 ± 2 minutes (1000 rpm in the TJ-6 centrifuge with the TH-4 rotor).

3.3.2.4 Pipette 12.0 ml of CVS Diluent into a CVS-labeled 16 x 125-mm glass tube with a flanged stopper.

3.3.2.5 Transfer an appropriate volume of the supernatant to the CVS-labeled tube so that the Working CVS contains between 12 and 50 50% lethal dose (LD_{50})/0.03 ml based on previous mouse titrations; mix by vortexing. Discard the pellet and any remaining supernatant in an appropriate manner.

3.3.2.6 The Working CVS is maintained in a bucket of ice until inoculated into mice. The Working CVS must be administered within 3 hours of preparation.

3.3.2.7 Preparation of CVS Back Titration on the day of challenge

1. Using a 25-ml pipette, dispense 9.0 ml of CVS Diluent into 3, 16 x 150-mm glass tubes with a Morton closure; label tubes 10^{-1} through 10^{-3}. Replace the Morton closure with a flanged stopper.

2. Transfer 1.0 ml of the Working CVS to the 10^{-1} tube with a 3-ml syringe and a 20-gauge needle; mix by vortexing.

3. With a new 3-ml syringe, repeat **Section 3.3.2.7(2)** for the remaining tubes, transferring 1.0 ml from the previous tube to the next tube; mix by vortexing.

4. The CVS Back Titration is maintained in a bucket of ice until inoculated into mice. The CVS Back Titration must be administered within 3 hours of preparation.

3.3.3 Preparation of CF-1 female mice

3.3.3.1 Mice are housed in a BL-2 animal room as defined by the NIH. Access should be limited to essential personnel.

3.3.3.2 For each Test Vaccine, 5 groups of 16 mice are recommended. Mice are housed in cages labeled with the Test Vaccine identification and stated dilution.

3.3.3.3 For the VRRV, 4 groups of 16 mice are required. Mice are housed in cages labeled as the VRRV and stated dilution.

3.3.3.4 For the CVS Back Titration, 4 groups of 10 mice are recommended. Mice are housed in cages labeled as the CVS Back Titration and the stated dilution. These mice are reserved until the day of CVS challenge.

3.4 Preparation of the sample

The starting dilution of a Test Vaccine should be based on the dilution, determined in the 5 replicate NIH tests at the time of the initiation of the host animal efficacy trial. The starting dilution should optimally protect 85-100% of the vaccinates. The starting dilution shall be stated in Part V of the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or if not stated, a dilution which meets the above criteria for a typical firm's Test Vaccine will be used by the CVB. A single NIH test per Test Vaccine is conducted. Sufficient volume of the Test Vaccine is required to prepare the dilution series required for 2 vaccinations. Starting with the dilution as determined from above, dilute the Test Vaccine similarly as stated in **Section 3.3.1.2.** Five dilutions/Test Vaccine or sufficient dilutions to meet the validity requirements in **Section 5.1** are inoculated into mice.

4. Performance of the test

4.1 Fill a 10-ml syringe and 25-gauge x 5/8-inch needle with 8.0 ml of diluted Test Vaccine or VRRV. **Note: The same syringe and needle may be used for a Test Vaccine or VRRV by starting with the most dilute followed by successively more concentrated dilutions.**

4.2 Inoculate 0.5 ml into each mouse intraperitoneally (IP), 16 mice/dilution, starting with the most dilute. Repeat for each dilution of a Test Vaccine and the VRRV using a new group of 16 mice caged according to dilution (**Section 3.3.3**).

4.3 At 7 ± 1 days postinoculation, repeat **Sections 4.1 through 4.2** for a second IP dose for the Test Vaccine and VRRV.

4.4 At 14 ± 1 days postinoculation, administer 0.03 ml of the Working CVS into each of the vaccinated mice and 10 control mice intracerebrally (IC), using a 0.5-ml tuberculin syringe with a 26-gauge x 3/8-inch needle. The Working CVS is inoculated through the frontal bone, midway between the right eye and the midline.

4.5 The CVS Back Titration is administered IC to the remaining 3 groups of 10 control mice. **Note: The same syringe and needle may be used if starting with the most dilute followed by successively more concentrated dilutions. The CVS Back Titration is not administered until all the vaccinated mice have been challenged.**

4.6 Observe all mice daily for 14 days postchallenge; record the number of deaths each day.

4.6.1 Mice dying prior to or on 5 days postchallenge are considered nonspecific deaths. Nonspecific deaths are not used in calculating the 50% effective dose (ED_{50}) of the Test Vaccine or VRRV.

4.6.2 Deaths occurring after 5 days postchallenge and those exhibiting clinical signs of rabies and exhibiting paresis, paralysis and/or convulsions are considered deaths caused by CVS challenge. These mice may be humanely euthanized and considered as deaths as outlined in 9CFR 117.4. Mice that survive the 14-day postchallenge observation period are euthanized at that time.

4.7 The ED_{50} of each Test Vaccine, VRRV, and the LD_{50} of the CVS are determined by the method of Spearman-Kärber as referenced in the fourth edition of the WHO, *Laboratory Techniques in Rabies*.

4.8 The relative potency (RP) of the Test Vaccine (TV) is determined by the formula:

$$RP = \frac{\text{reciprocal } ED_{50} \text{ of TV}}{\text{reciprocal } ED_{50} \text{ of VRRV}} \times \frac{\text{dose of TV}}{\text{dose of VRRV}}$$

ED_{50} of TV = 1:90
ED_{50} of VRRV = 1:70
90/70 = 1.29 RP/ml

4.9 The RP value may be expressed in international units (IU) by multiplying the RP by the IU/ml of the VRRV. For a VRRV with a known value of 1.0 IU/ml, in the above example:

1.29 X 1.0 IU/ml = 1.29 IU/ml.

5. Interpretation of the test results

5.1 For a valid test:

5.1.1 At least 70% (μ70%) of the mice receiving the most concentrated dilutions of VRRV and the Test Vaccine must survive (e.g., 11 out of 16 mice).

5.1.2 At least 70% (μ70%) of the mice receiving the least concentrated dilution of VRRV and the Test Vaccine must die (e.g., 11 out of 16 mice).

5.1.3 The CVS Back Titration must show that between 12 and 50 LD_{50} was administered.

5.1.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.2 The minimum RP value for a **SATISFACTORY** Test Vaccine is determined by the results of the host animal immunogenicity serial as required by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.209, and contained in an APHIS filed Outline of Production.

5.3 If the Test Vaccine does not meet the minimum RP value contained in an APHIS filed Outline of Production, as determined from the initial NIH test, the Test Vaccine may be retested. If the Test Vaccine is retested, 2 independent NIH tests shall be conducted. A geometric mean RP of all 3 retests is used to evaluate the Test Vaccine.

6. Report of test results

Test results are reported as the RP/ml.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.209, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Richmond JY, McKinney RW (eds). Biosafety in microbiological and biomedical laboratories. US Department of Health and Human Services, US Government Printing Office, Washington. 1993; p. 177.

7.3 Wilbur LA, Aubert MFA. The NIH test for potency. In *Laboratory Techniques in Rabies, 4th ed.*, Meslin, FX, MM Kaplan, and H Koprowski, eds. World Health Organization, Geneva. 1996, Chpt 37.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **1.2** "Key Words" has been deleted.
- **3.3.1.2** The example of the fivefold dilution series starting at 1:10 was expanded.
- **3.4** has been reworded for clarification.
- **4.6.2** has been reworded for clarification.
- **5.2** has been reworded for clarification.
- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.

9. Addendum

A template for calculating the ED_{50}, LD_{50}, and the RP is available upon request from the CVB. The template calculates the values as defined by the fourth edition of the WHO, *Laboratory Techniques in Rabies*.